Complement anaphylatoxin receptors C3aR and C5aR are required in the pathogenesis of experimental autoimmune uveitis

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ABSTRACT
Recent studies have suggested that reagents inhibiting complement activation could be effective in treating T cell mediated autoimmune diseases such as autoimmune uveitis. However, the precise role of the complement anaphylatoxin receptors (C3a and C5a receptors) in the pathogenesis of autoimmune uveitis remains elusive and controversial. We induced experimental autoimmune uveitis in mice deficient or sufficient in both C3a and C5a receptors and rigorously compared their retinal phenotype using various imaging techniques, including indirect ophthalmoscopy, confocal scanning laser ophthalmoscopy, spectral domain optical coherence tomography, topical endoscopic fundus imaging, and histopathological analysis. We also assessed retinal function using electroretinography. Moreover, we performed Ag-specific T cell recall assays and T cell adoptive transfer experiments to compare pathogenic T cell activity between wild-type and knockout mice with experimental autoimmune uveitis. These experiments showed that C3a receptor/C5a receptor-deficient mice developed much less severe uveitis than did control mice using all retinal examination methods and that these mice had reduced pathogenic T cell responses. Our data demonstrate that both complement anaphylatoxin receptors are important for the development of experimental autoimmune uveitis, suggesting that targeting these receptors could be a valid approach for treating patients with autoimmune uveitis.


Introduction
Autoimmune uveitis, a major cause of blindness, has an unclear etiology, and many patients fail to respond to treatment [1]. EAU, induced by IRBP peptide immunization in mice, has been widely used to study the pathogenesis of autoimmune uveitis and to develop new therapies for this disease [2]. Adoptive transfer of activated T cells from animals with EAU is sufficient to induce disease in naive recipients [3, 4], suggesting that this disease is mainly T cell mediated. It has been established that autoreactive Th1 and Th17 cells are the primary mechanisms underlying the pathogenesis of EAU [5].

Although complement has been thought to function as an effector system for antibodies [6], recent accumulating evidence indicates that in addition to its conventional roles in the innate immune system, complement is also important in directly regulating T cell responses in the adaptive immune response through different mechanisms [7]. Among them, the complement receptors C3aR and C5aR have been found to synergically augment T cell responses in several studies in which C3aR and C5aR—whether on APCs or on T cells—have been shown to be required for a robust T cell response [8–12]. However, contradictory reports have shown that a deficiency of C5aR on dendritic cells actually promotes pathologic Th17 responses and that antibody blockade of C5aR enhances Th17 cell numbers in an experimental model of asthma [13].

Several lines of previous studies have suggested that activated complement contributes significantly to the pathogenesis of EAU [14–16]. Also, at least some of these studies [15, 16] have suggested that complement does so by regulating T cell responses, implying an important role for C3aR and C5aR in the development of EAU. However, surprisingly, a recent brief report [17] suggested that C3aR and C5aR are not required, because no retinal histopathological difference was noted between WT and C3aR/C5aR KO mice after they were immunized with IRBP1–20 peptide to induce EAU.

To understand further the mechanism by which complement regulates the pathogenesis of EAU and to clarify the importance of the complement anaphylatoxin receptors in the development of EAU, we compared the disease severity in C3aR/C5aR KO mice and WT mice with EAU. In our experiments, IRBP1–20 immunization resulted in a mild and inconsistent disease with incomplete penetrance, making the data difficult to analyze. In the present

Abbreviations: AF = autofluorescence, C3aR = C3a receptor, C5aR = C5a receptor, CC = choroidal complex, cSLO = confocal scanning laser ophthalmoscopy, EAU = experimental autoimmune uveitis, EFQ = electroretinography, IF = infrared, IFPB = interphotorceptor retinoid-binding protein, KO = knockout, RFDF = red free dark field, RFPE = retinal pigment epithelium, SD-OCT = spectral domain optical coherence tomography, TEFI = topical endoscopic fundus imaging, VFI = viretretinal, WT = wild-type

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study, we used a recently defined IRBP61-470 peptide, which is superior to the IRBP1-290 peptide in terms of EAU penetrance and scoring [18]. We monitored EAU development by indirect ophthalmoscopy and examined the fundus in detail using cSLO, SD-OCT, TEFI, and retinal histopathological analysis. We also evaluated retinal function using ERG and assessed Ag-specific T cell responses using recall assays and T cell adoptive transfer experiments. In contrast to the data obtained with the IRBP1-290 model of EAU, our data have demonstrated that these 2 complement anaphylatoxin receptors are important in the pathogenesis of EAU, because they are required for the development of pathogenic T cells.

MATERIALS AND METHODS

Animals
C57BL/6 WT and C3aR/C5aR double-KO mice (backcrossed with C57BL/6j mice for >12 generations, 8–12 wk old) were developed and have been described in previous reports [10, 11, 19]. These mice were maintained under pathogen-free conditions in the animal facility of Lerner Research Institute, Cleveland Clinic (Cleveland, OH, USA). The Institutional Animal Care and Use Committee of Cleveland Clinic approved all the procedures involving mice, all of which were performed in accordance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and institutional guidelines.

Induction of EAU
For induction of EAU, the WT and KO mice were immunized by subcutaneous injection of 200 μl of an emulsion containing 200 μg of human IRBP61-470 peptide LAQGAYRTAVDLESLASQLT [20] (custom synthesized by GenScript USA Inc., Piscataway, NJ, USA) and 250 μg of Mycobacterium tuberculosis H37Ra in complete Freund’s adjuvant (Difco Laboratories, Inc., Detroit, MI, USA). The emulsion was injected into each thigh (50 μl each) and at the base of the tail (100 μl). A single dose of 500 ng of pertussis toxin (List Biologic Laboratories, Inc., Campbell, CA, USA) was given by intraperitoneal injection after immunization on the same day.

Clinical and histopathological scoring of EAU
The mice were examined daily for clinical signs of EAU using a binocular indirect ophthalmoscope (Keeler Instruments, Inc., Broomall, PA, USA). The pupils were dilated using a mixed ophthalmic solution of 0.5% tropicamide and 1.25% phenylephrine hydrochloride. The disease severity was scored on a scale of 0–4, according to published criteria [2].

For retinal histopathological evaluation, the mice were euthanized, and the whole eyes were collected on d 14 or 20 after immunization and immersed in 10% formaldehyde in PBS buffer for fixation. The fixed tissues were embedded in paraffin and processed. Sections of 5 μm were cut through the pupil and optic nerve axis and stained with H&E. Retinal histopathological changes were graded in a blinded fashion at the National Eye Institute using 4 sections for each eye, according to previously published scoring criteria [2].

Retinal imaging
Retinal imaging was performed using cSLO, SD-OCT, and TEFI in accordance with published protocols after general anesthesia [21, 22]. cSLO (Heidelberg Retina Angiograph II; Heidelberg Engineering, Carlsbad, CA, USA) collects both reflectance and fluorescence information from the posterior segment of the mouse [23]. Equipped with a 55° wide-field objective lens, the system provides a fundus cSLO image with a lateral resolution of 850 μm (at 50% modulation). The clinical scores and ERG results were assessed by repeated-measures ANOVA. Scores from the 2 eyes of 1 mouse were averaged and considered a single event, as an independent variable. Student’s t test was used for 2 sets of data. Data are expressed as the mean ± SD, except for the number of mice.
results, which are presented as the mean ± SEM. Statistical significance was set at P < 0.05.

RESULTS

Deficiency of C3aR/C5aR results in significantly reduced EAU clinical scores

We first immunized 8 WT and 8 KO mice with IRBP<sub>651–670</sub> peptide to induce EAU. For the next 14 d, we examined the retina by indirect ophthalmoscopy and recorded the clinical scores daily, in accordance with previously published scoring criteria [2]. These experiments showed that although WT mice developed retinal inflammation, KO mice were protected, which was indicated by the significantly lower clinical scores for the KO mice than for the WT mice (Fig. 1A). We repeated the experiment 2 more times with 5 mice per group and monitored the mice until d 19. These experimental results showed that the KO mice consistently developed less severe EAU than the WT mice (Fig. 1B and C). These data indicate that the anaphylatoxin receptors C3aR/C5aR are indeed required for the pathogenesis of EAU.

Retinal imaging assessments of C3aR/C5aR-sufficient vs. C3aR/C5aR-deficient mice with uveitis

In addition to using indirect ophthalmoscopy examinations to assess retinal inflammation, we evaluated EAU severity in one half of the WT and KO mice on d 14 by cSLO and SD-OCT, 2 imaging techniques widely used in the clinic to evaluate uveitis in patients [31, 32]. We also used TEFI to obtain visible light fundus images for comparison. In agreement with previous results obtained from indirect ophthalmoscopy examinations, these imaging assessments revealed the consistent presence of multiple retinal pathologic features in the WT but not in the KO mice (Fig. 2), including 1) numerous chorioretinal lesions observed by TEFI (Fig. 2A vs. B), 2) increased AF features observed using AF-cSLO in the inner retina (Fig. 2D vs. H) in close proximity to the VRI, adjacent to the retinal vasculature and nerve fibers, and in the ganglion cell layer, 3) asymmetric, hyperreflective features (Fig. 2E vs. I) observed using IR-cSLO that circumvent and/or extend from the optic nerve, 4) elongated AF lesions observed in the outer (Fig. 2F vs. J) retina by AF-cSLO, and 5) reflective foci within the vitreous chamber and prominent retinal infoldings observed by SD-OCT (Fig. 2K). These lesions were often interconnected when closer to the optic nerve and occasionally formed a semicontiguous ring around it (Fig. 2A and F). Beyond the initial ring, most lesions were isolated as individual spots and/or retinal infoldings. SD-OCT imaging also showed prominent changes to the outer retinal architecture in 75% of the WT mice examined, including complete loss of external limiting membrane and inner segment/outer segment bands. Hyperreflective retinal infoldings originate from the RPE apical surface, extending into the outer nuclear layer, and displacing inner retina laminae including outer plexiform layer, inner nuclear layer, and inner plexiform layer. KO mice rarely exhibited any of these features, and if they did, the features were substantially less in magnitude than in the WT mice (Fig. 2B, G–J, and L).

Anaphylatoxin receptors C3aR/C5aR reduce ERG sensitivity in the uveitic mouse retina

ERG is another sensitive assay to evaluate EAU severity from a different perspective [33, 34]. We used strobe flash ERGs to compare the outer retinal function of WT and KO mice for retinal injury assessment 14 d after immunization as a complementary approach for the imaging assays used. These experiments showed that under every intensity of flash stimulus, all ERG amplitudes of both a- and b-waves under the dark-adapted condition and the b-wave under the light-adapted condition were all significantly lower in the WT mice with EAU than in the KO mice with EAU (Fig. 3). This finding suggests that the function of photoreceptors and bipolar cells in both rod and cone pathways of WT mice was damaged more severely than in KO mice, a result consistent with the augmented EAU severity in WT mice, which was assessed by indirect ophthalmoscopy, cSLO, SD-OCT, and TEFI (Figs. 1 and 2).

Anaphylatoxin receptors C3aR/C5aR increase histopathological scores in EAU mice

We next evaluated EAU severity by retinal histopathological analysis. At the end of each experiment, the eyes were harvested, and paraffin-embedded ocular sections were prepared for H&E staining. The slides were examined and scored in a blinded fashion at the National Eye Institute in accordance with the established mouse EAU histopathological scoring criteria [2]. These examinations found that in all 3 experiments, the KO mice displayed significantly reduced histopathological scores compared with the WT mice after EAU induction (Fig. 4), in accordance with all previous retinal imaging and function assays (Figs. 2 and 3).

Figure 1. C3aR/C5aR KO mice have decreased clinical scores in EAU. WT and KO mice were immunized to induce EAU, and the degree of manifestation of clinical uveitis was examined using a binocular indirect ophthalmoscope (n = 8 in each group for the first experiment, n = 5 in each group for the second experiment, and n = 5 in each group for the third experiment). Clinical scores from the 2 eyes of each mouse were averaged and considered as a single event. *P < 0.05. DKO, double knockout; EXP., experiment.
Adoptive transfer of in vitro–activated C3aR/C5aR-deficient T cells induces ameliorated EAU

C3aR and C5aR are present on T cells, especially activated T cells [11], and on APCs, such as dendritic cells and macrophages [35]. To assess the function of the T cells from WT and C3aR/C5aR KO mice with EAU, we compared the efficacy of the same number of WT and KO T cells in inducing retinal inflammation in naïve recipient mice. Using an established protocol [36, 37], we activated the IRBP-specific T cells in vitro by culturing splenocytes from WT and KO mice with EAU using the same concentrations of IRBP peptide, together with IL-23, for 3 d. We then enriched the activated (blasted) T cells by Ficoll centrifugation and adoptively transferred a fixed number of the in vitro–activated (blasted) WT or KO T cells into naïve recipient mice. We evaluated EAU development by indirect ophthalmoscopy. Adoptive transfer of activated WT T cells induced more severe retinal inflammation than did the same number of KO T cells (Fig. 5), showing that C3aR/C5aR-deficient T cells are less potent than WT T cells for inducing EAU.

Immunologic assays for IRBP-specific Th1 and Th17 responses

In addition to the clinical and histopathological evaluation of disease, we performed Ag-specific recall assays to assess the pathologic IRBP-specific Th1 and Th17 responses in both WT and KO mice with EAU [15]. After incubating splenocytes from WT and KO mice with EAU in the presence of the immunizing IRBP peptide or a nonrelevant peptide, we measured the levels of IFN-γ and IL-17 (signature cytokines for Th1 and Th17 response) in the culture supernatants using conventional ELISA. In keeping with the markedly reduced clinical, functional, and histopathological manifestations of the retinas, the KO mice consistently showed significantly reduced IRBP-specific IFN-γ and IL-17 responses compared with the responses of the WT mice (Fig. 6), suggesting that C3aR and

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**Figure 2.** Representative ocular pathology examples documented by in vivo imaging. Hyperreflective lesions were observed in WT (A) but not KO (B) mice using TEFI cSLO images further isolated these features as hyperreflective and/or AF changes to both the inner (C–H) and outer (E–J) retina. Numerous foci can be seen adjacent to the retinal vessels and in the avascular space of WT (D, arrows) vs. KO mice (H, downward-pointing arrows). Hyperreflective streaks (arrows) can be observed emanating from the optic disk at ~30°, 150°, and 270°. Other hyperreflective areas (circled) can be seen (E), which were rarely observed (circled) in KO mice (J). AF-cSLO (F) revealed AF lesions (arrows) previously shown by TEFI (A). KO mice (J) were void of AF lesions resembling those observed in WT mice (F). SD-OCT B-scans ranging from the posterior lens to beyond the choroid revealed more reflective foci in the WT mice (K, down arrows) compared with those in the KO mice (L). The foci likely represented clusters of active inflammatory cells or cellular debris in the vitreous cavity (VC) in close proximity to the VRI (K). SD-OCT image field of view = 1 mm × 1.5 mm.
C5aR are required for development of a robust autoreactive T cell response necessary to induce EAU.

**DISCUSSION**

In the present project, using WT and C3aR/C5aR KO mice, we studied the significance of the complement anaphylatoxin receptors in the pathogenesis of immunization-induced EAU. Using multiple imaging techniques, including indirect ophthalmoscopy, cSLO, SD-OCT, TEFI, and retinal histopathological analysis, we consistently found that KO mice had less severe retinal inflammation than WT mice after immunization. These imaging results are consistent with the results from ERG and immunologic assays, which showed better preservation of outer retina function and less-intense IRBP-specific Th1/Th17 responses in KO mice than in WT mice, suggesting that both C3aR and C5aR are important in the pathogenesis of EAU.

Complement is an important part of the innate immune system, which primarily serves as the body’s first defense against pathogen invasion [38]. After activation, the complement-activation product C3b and its derivatives bind to the target cells, facilitating phagocytosis, a process known as opsonization. Complement activation also leads to the formation of membrane attack complexes, which directly assault the target cells. In addition to these cell-bound complement-activation products, anaphylatoxins (including C3a and C5a) are produced and released into the fluid phase during complement activation. After binding to their respective receptors C3aR and C5aR, both...
of which are G-protein–coupled receptors, C3a and C5a can activate and recruit leukocytes to the site of complement activation to facilitate inflammation and clear up the invading pathogens [39]. In addition to these conventional roles of complement in fighting infections as a part of the innate immune system, accumulating evidence suggests that complement is also important in regulating T cell responses from the adaptive immune system. C3aR and C5aR have received much attention as key players for complement to directly regulate T cell responses, because both APCs and T cells express these anaphylatoxin receptors [40]. It has been demonstrated that during APC and T cell interaction, both partners locally produce complement components, and complement activation occurs inside or near the immune synapse to produce C3a and C5a [11]. These locally produced C3a and C5a components, after binding to C3aR and C5aR on APCs, promote the surface expression of MHC and costimulatory molecules, facilitating priming [10]. At the same time, they also bind to C3aR and C5aR on T cells; this binding inhibits activation-induced T cell apoptosis, facilitates Th1 and Th17 effector T cell differentiation [11], and inhibits the development of the immunosuppressive Foxp3+ T-regulatory cells [19, 41], leading to more robust T cell responses.

EAU has been established as a primarily T cell mediated model for autoimmune posterior uveitis [2]. Although the conventional role of complement (e.g., membrane attack complex formation and opsonization) is not likely to significantly contribute to the pathogenesis of the disease, it is possible that complement is still important through its newly discovered T cell regulatory activities. It has been reported that C3-deficient mice and mice overexpressing a soluble form of the complement inhibitor Crry show signs of less severe EAU after immunization as assessed by histopathologic examination [14]. However, the potential effect of these genetic changes on the immune response associated with EAU was not examined in this study. We have shown that mice deficient in the cell-surface complement inhibitor CD55, in which local complement activation is therefore insufficiently controlled, had enhanced retinal inflammation in EAU and augmented IRBP-specific Th1/Th17 responses [15]. These results are consistent with previous reports showing that in the absence of CD55, augmented levels of C3a and C5a are produced that interact with C3aR and C5aR on APCs and/or T cells, leading to enhanced T cell responses [10, 42]. In addition, it has been demonstrated that treating mice with an anti-C5 mAb, which inhibits C5 activation and C5a release, reduced the disease severity of EAU [16]. All these data associating complement with the pathogenesis of EAU, and other studies showing the importance of C3aR and C5aR in regulating T cell responses in other disease models [43], strongly suggest that C3aR/C5aR would be required for the development of EAU. Moreover, the results of studies using a similar ocular disease model, experimental autoimmune anterior uveitis, also strongly suggest that complement and, potentially C3a/C5a, should be integrally involved in autoreactive T cell response development and ocular inflammation [44, 45]. Our data support and expand this interpretation through imaging, functional, histopathological, and immunologic evidence that C3aR/C5aR KO deficiency reduces disease severity and the associated immunologic effector responses.

Surprisingly, a recent brief report has suggested that C3aR/C5aR KO mice with the same C57BL/6 background did not show any defect in developing EAU compared with control WT mice [17]. The exact reasons behind this apparent discrepancy are unclear; however, housing conditions could have played a role, because environmental factors, including the gut microbiome, has been found to affect the pathogenesis of many disease

Figure 4. C3R/C5aR KO mice showed reduced histopathological scores in EAU. Paraffin-embedded ocular sections were prepared and stained by H&E. The slides were examined in a blinded fashion at the National Eye Institute, and histopathological scores were assigned. A) Histopathological scores of WT and KO mice in EAU from 3 independent experiments. Each dot represents the average score of 2 eyes from 1 mouse. *P < 0.05. B) Representative retinal histologic images of WT and KO mice in EAU. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

Figure 5. C3aR/C5aR KO T cells have defect in inducing EAU after adoptive transfer. The same numbers of in vitro restimulated and activated (blasting) T cells from WT and KO EAU mice were adoptively transferred into naive recipient mice. The respective clinical scores were recorded (A), and the representative retina images of these adoptively transferred mice are presented (B) (n = 10 in each group). Data are presented as mean ± SEM. *P < 0.05.
models, including EAU [46–48]. Also, the histopathological analysis was the only assay used to compare the severity of EAU between WT and KO mice for the whole study [17]. Thus, any effects on disease (onset, kinetics) up to that point would have been missed. Finally, IRBP1–20 is a very weak epitope, resulting in borderline disease scores and incomplete penetrance, making data in which disease inhibition is expected difficult to interpret [2].

In the present study, in addition to rigorously examining the retina using various imaging and histopathological techniques, we evaluated retinal function using ERG and autoreactive T cell activities by Th1/Th17 recall assays and T cell adoptive transfer experiments. Moreover, we used the IRBP651–670 peptide, a more recently discovered epitope that is uveitogenic in C57BL/6J mice, and induces more robust and reproducible EAU in WT C57BL/6J mice than does the IRBP1–20 peptide [18]. This new immunization protocol should be valuable in overcoming the shortcomings of the previous protocol using the IRBP1–20 peptide for EAU studies, especially for conditions in which reduced EAU severity is anticipated in the experimental subjects.

In conclusion, consistent with accumulating evidence suggesting that C3aR and C5aR are positive regulators for pathogenic T cell development, using different imaging studies, function studies, and immunologic assays to study EAU in WT and C3aR/C5aR KO mice, we found that these complement receptors are required in the pathogenesis of EAU. These findings identify C3aR and C5aR as important receptors necessary for the full development of EAU and the associated Th1 and Th17 effector responses, suggesting that antagonists of these complement receptors should be examined for their potential as new drugs for the treatment of autoimmune uveitis.

AUTHORSHIP
L.Z., B.A.B., M.Y., and C.-C.C. performed the experiments, analyzed data, and edited the manuscript. N.S.P., J.F., X.Z., and R.R.C. analyzed data and reviewed and/or edited the manuscript. F.L. designed the experiments, analyzed the data, and prepared the manuscript.

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DISCLOSURES
The authors declare no conflicts of interest.

REFERENCES

Figure 6. C3aR/C5aR mice show reduced Ag-specific Th1 and Th17 responses in EAU. Splenocytes were incubated with or without 10 μg/ml IRBP peptide or the same concentration of ovalbumin (OVA)323–339 peptide for 3 d. The levels of IFN-γ, a signature cytokine for Th1 cells, and IL-17, a signature cytokine for Th17 cells, were measured by ELISA. Results shown are from 3 independent experiments. *P < 0.05. EXP., experiment.


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